Differentiation of Human Embryonic Stem Cells into Clinically Amenable Keratinocytes in an Autogenic Environment

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Human embryonic stem cells (hESCs)-derived keratinocytes hold great clinical and research potential. However, the current techniques are hampered by the use of xenogenic components that limits their clinical application. Here we demonstrated an efficient differentiation of H9 hESCs (H9-hESCs) into keratinocytes (H9-Kert) with the minimum use of animal-derived materials. For differentiation, we established two microenvironment systems originated from H9-hESCs (autogenic microenvironment). These autogenic microenvironment systems consist of an autogenic coculture system (ACC) and an autogenic feeder-free system (AFF). In addition, we showed a stage-specific effect of Activin in promoting keratinocyte differentiation from H9-hESCs while repressing the expression of early neural markers in the ACC system. Furthermore, we also explained the effect of Activin in construction of the AFF system made up of extracellular matrix similar to basement membrane extracted from H9-hESC-derived fibroblasts. H9-Kert differentiated in both systems expressed keratinocyte markers at mRNA and protein levels. H9-Kert were also able to undergo terminal differentiation in high Ca^{2+} medium. These findings support the transition toward the establishment of an animal-free microenvironment for successful differentiation of hESCs into keratinocytes for potential clinical application.

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INTRODUCTION

Skin is one of the largest organs of the body and acts as the primary barrier between the external environment and the deeper tissues. However, conditions such as foot ulcers, skin defects, and massive burns often result in open wounds and require rapid surgical closure with appropriate materials (Izumi *et al.*, 2003). Surgical closures with autologous epidermal grafts are ideal but have several disadvantages including donor site morbidity, time constraint, and limited cell yield from primary explanted keratinocytes. Alternatively, commercially available grafts with allogeneic primary cells can be used, but because of the immune response of the patients, these grafts suffer the risks of potential rejection (Metcalfe and Ferguson, 2007; Lemaître *et al.*, 2011). For the last decade, human embryonic stem cells (hESCs) have been suggested as a substitute cell source for keratinocytes. hESCs are derived from the inner cell mass of human blastocysts (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000). They possess the unique property of unlimited proliferation and differentiation into all types of somatic cells originated from three germ layers (Thomson *et al.*, 1998). Furthermore, Guenou *et al.* in 2009 have reported that keratinocytes derived from hESCs express low major histocompatibility complex class I (HLA-ABC) as compared with primary keratinocytes. Therefore, hESC-derived keratinocytes have great potential in regenerative medicine and dentistry as a limitless cell source for rapid tissue replacement in patient treatment (Metallo *et al.*, 2007; Guenou *et al.*, 2009; Lemaître *et al.*, 2011).

Green *et al.* pioneered studies of keratinocyte differentiation from hESCs in 2003. Since then, various groups have reported

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Abbreviations: ACC system, autogenic coculture system; AFF system, autogenic feeder-free system; DSFM, defined keratinocyte serum-free medium; ECM, extracellular matrix; ES medium, embryonic stem cell medium; FBS, fetal bovine serum; H9-ebF, H9-hESC-derived fibroblast; H9-hESC, H9-human embryonic stem cell; H9-keratinocyte, H9-hESC-derived keratinocyte; H9-Kert^{ACC}, H9-hESC-derived keratinocyte in an autogenic coculture system; H9-Kert^{AFF}, H9-hESC-derived keratinocyte in an autogenic feeder-free system; HK, human keratinocyte (HaCaT cell line); RA, retinoic acid; RT, room temperature

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hESCs' differentiation into keratinocytes (Aberdam 2004; De Luca *et al.*, 2006; Iuchi *et al.*, 2006; Ji *et al.*, 2006; Metallo *et al.*, 2008; Guenou *et al.*, 2009; Hewitt *et al.*, 2009). However, to date, the strategies required for the induction of hESCs toward the keratinocyte lineage involve the use of xenogeneic microenvironments such as feeder cells and/or extracellular matrix (ECM). The presence of these xenogeneic components in a culture milieu limits the clinical application of hESCs because of the potential risk of transmission of animal pathogens and viral or bacterial infections (Cobo *et al.*, 2005; Sjögren-Jansson *et al.*, 2005; Skottman and Hovatta, 2006; Rajala *et al.*, 2010). Alternatively, it has been shown that the exposure of hESCs to animal components in culture systems causes higher expression of immunogenic agents (e.g., *N*-glycolylneuraminic acid and Neu5Ac) into hESCs (Martin *et al.*, 2005; Heiskanen *et al.*, 2007).

Clearly, there is a need to eradicate or at least minimize the exposure to xenogeneic components in the differentiation protocols to enhance the clinical application of hESC-derived keratinocytes (Mallon *et al.*, 2006). Therefore, in this study, we aim to minimize the use of animal components in the differentiation protocol to get a pure and expandable population of keratinocytes from hESCs in the autogenic microenvironment instead of the xenogeneic microenvironment.

RESULTS AND DISCUSSION

Characterization of H9-ebF feeders for the autogenic coculture system (ACC) system

To achieve our aim, we first differentiated H9-hESCs into fibroblasts (H9-ebF). The importance of fibroblasts in keratinocyte morphogenesis has been studied by few researchers (Mackenzie *et al.*, 1993; el-Ghalbzouri *et al.*, 2002; El Ghalbzouri *et al.*, 2002). H9-ebFs were grown and characterized for 10 passages. The expression of the pluripotency marker Oct4 was observed from passages 1 to 4 on reverse transcriptase–PCR electrophoretic gel at 1.5 seconds of UV exposure (Figure 1a). Therefore, we assumed that a portion of H9-ebFs at passages 1 to 4 were not fully differentiated and were inappropriate for setting up the ACC system. However, passages 5 to 10 were further characterized for the expression of Vimentin and Prolyl 4-hydroxylase to confirm their fibroblastic lineage (Figure 1b).

In addition, it has been shown that fibroblasts are responsible for secreting different growth factors like keratinocyte growth factor-I, which in particular, has an important role in keratinocyte morphogenesis (Fusenig *et al.*, 1994; Maas-Szabowski *et al.*, 1999). Therefore, to further optimize conditions for keratinocyte differentiation, the expression of keratinocyte growth factor-I was analyzed from passages 5 to 10 of H9-ebF. The level of keratinocyte growth factor-I was found to be the highest in H9-ebF-passage 8 (P8) (Figure 1c). H9-ebF P8 was further characterized for fibronectin expression, which is normally produced by cultured fibroblasts (McKeown-Longo *et al.*, 1984) (Figure 1d). Correspondingly, we found no expression of epithelial precursor marker K18 and/or epidermal marker K14 in H9-ebF P8 (Figure 1d and e).

Establishment of the ACC system and medium optimization

For the establishment of the ACC system, H9-hESCs were cocultured with inactivated H9-ebF P8. H9-hESCs and H9-ebFs required different culture conditions. Therefore, it was necessary to optimize the culture milieu suitable for both cell types. The ACC system was kept in three different medium conditions for 5 days (F12 and Dulbecco medium), defined keratinocyte serum-free medium (DSFM), and embryonic stem cell medium (ES medium, without fibroblast growth factor). Viability of cocultured cells was tested by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)-cell viability assay. Viability assay results showed that only F12 and Dulbecco medium and ES medium were able to support the ACC system with no significant difference between them. Correspondingly, we found significantly less viable cells in the ACC system kept in DSFM compared with other groups (Figure 2a). Thus, we selected F12 and Dulbecco medium for keratinocyte differentiation in the ACC system.

Retinoic acid (RA) induces neuroectodermal differentiation of H9-hESCs in the ACC system

For the induction of keratinocyte differentiation, we used RA as previously reported (Metallo *et al.*, 2008). It has been shown previously that RA induces ΔNp63 expression through the RA receptor (Schuldiner *et al.*, 2000; Chen and Lohnes, 2005). ΔNp63 is supposed to be a well-known marker for epidermal progenitors and an essential transcription factor for keratinocyte differentiation (Mills *et al.*, 1999; Yang *et al.*, 1999; McKeon, 2004).

For dose optimization of RA, three different concentrations (0.1, 0.5, and 1 μM) were applied. However, in contrast to the findings of Metallo *et al.*, our flow cytometry results showed that application of 0.5 μM of RA induces significantly higher ΔNp63 (+) cells compared with other concentrations of RA in the ACC system (Figure 2b).

To analyze the temporal effect of RA, the ACC system was treated with 0.5 μM of RA until day 10 and day 20, respectively. Real-time reverse transcriptase–PCR results showed that the continuous exposure treatment for 20 days resulted in higher expression of ΔNp63. However, we also found a significant increase in the expression of neural markers (Pax6 and Sox1) (Figure 2c). A possible explanation for the high neural contamination could be the expression of fibroblast growth factors by fibroblasts, which induce neural differentiation of hESCs through the extracellular signal–regulated kinase 1/2–poly (ADP-ribose) polymerase 1 pathway (Yoo *et al.*, 2011). Therefore, it was essential to inhibit the neural differentiation to increase the purity of keratinocyte population from H9-hESCs.

Effect of Activin in keratinocyte differentiation in the ACC system

The results described above led us to focus on neural inhibition. Therefore, in this study, we postulate that Activin, which is a member of the transforming growth factor-β family, may be a potent inhibitor of the neural pathways. Previously, Activin has been used to maintain mouse embryonic stem cell and hESC pluripotency (Beattie *et al.*, 2005; James *et al.*, 2005;

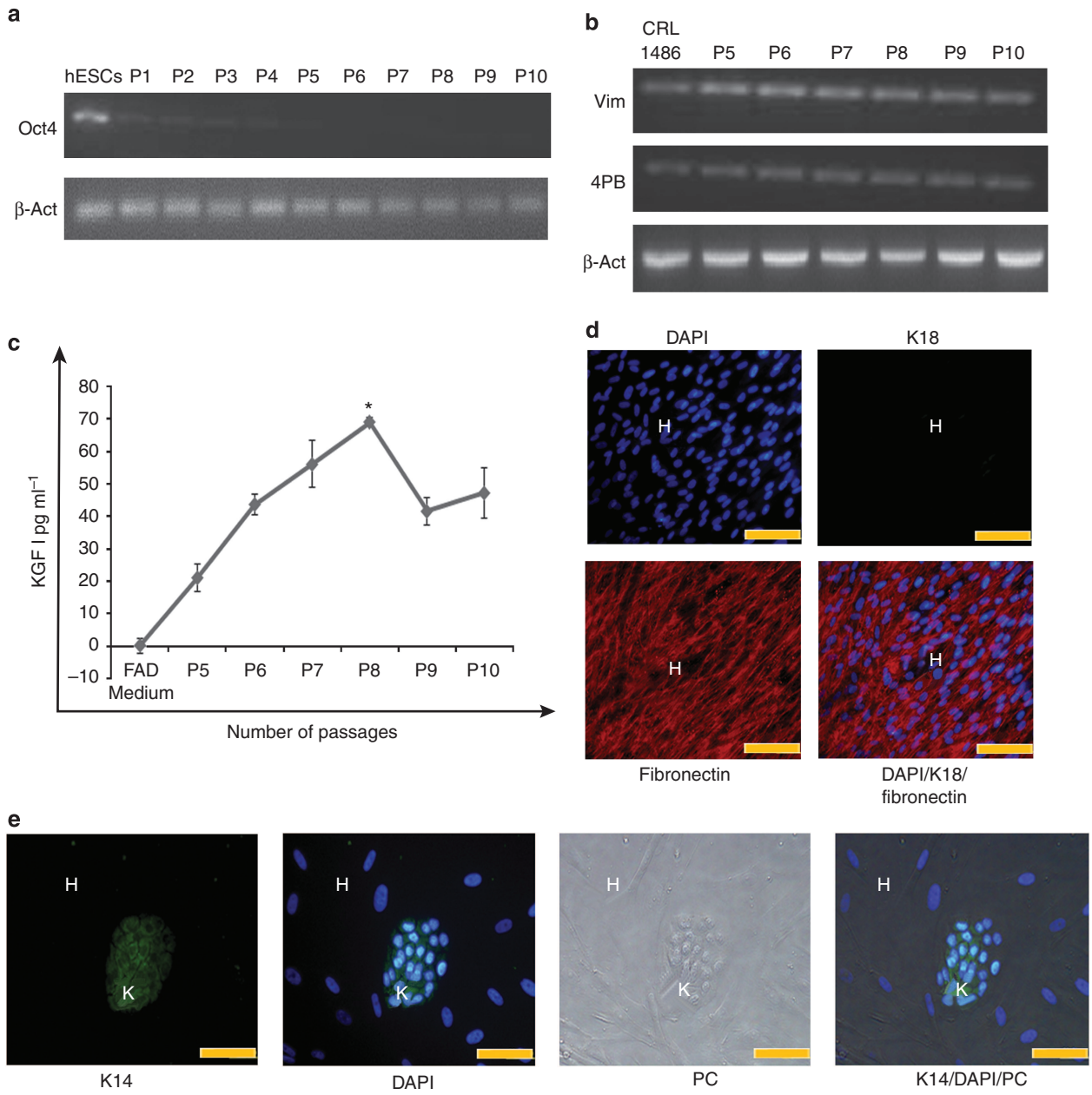

Figure 1. Characterization of H9-ebF. (**a**, **b**) Qualitative analysis of gene expression profile of (**a**) pluripotency marker and (**b**) fibroblast markers. β-Actin was taken as the loading control and run on a separate gel. (**c**) Results of ELISA show the KGF-I secretion by P 5–P 10 of H9-ebF. Error bars indicate the mean ± SEM ($n = 3$). *$P < 0.05$ versus all groups. (**d**, **e**) Immunofluorescence staining shows the expression of (**d**) K18 and fibronectin by H9-ebF passage 8 (scale bar = 100 μm), (**e**) K14 by HaCaT cells–H9-ebF passage 8 in coculture (scale bar = 50 μm). β-Act, β-actin; CRL-1486, human embryonic palatal mesenchymal cell line; DAPI, 4,6-diamidino-2-phenylindole; FAD, F12 and Dulbecco medium; H, H9-ebF; hESC, human embryonic stem cell; H9-ebF, H9-hESC-derived fibroblast; K, HaCaT cells; KGF-I, keratinocyte growth factor-I; P, passage; 4PB, prolyl 4-hydroxylase; PC, phase contrast; Vim, vimentin.

Vallier *et al.*, 2005) by inhibition of default neural differentiation (Camus *et al.*, 2006; Patani *et al.*, 2009). Therefore, it is of interest to find the effect of dose modulation and temporal applications of Activin on inhibition of neural differentiation alone, without effecting keratinocyte induction. However, the effect of Activin on keratinocyte differentiation in the presence of RA has never been reported.

To analyze the temporal effect, Activin was applied for 3 days at different time points (day 1–day 11) of differentiation. We observed different effects of Activin when applied at different time points (Figure 3a and b). The early application of Activin at day 1 and day 2 inhibits the neuroectodermal differentiation of H9-hESCs by keeping the cells in a pluripotent state. Interestingly, we found that the application of Activin at day 3, day 4, day 5, day 6, day 7, and day 8

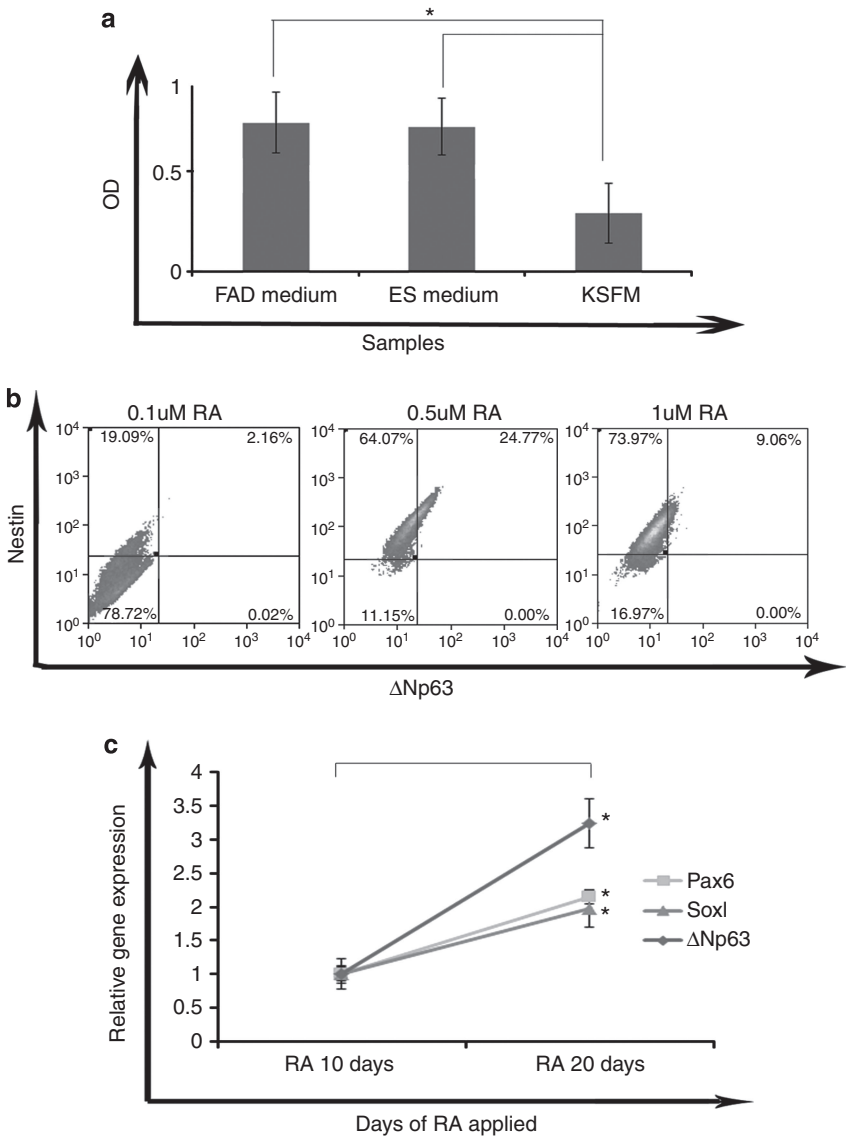

Figure 2. Optimization of *in vitro* conditions. (**a**) 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay results show the cell viability in the ACC system in different medium conditions at day 5. Error bars indicate the mean ± SEM ($n = 3$); *$P < 0.05$ versus "KSFM" sample. (**b**, **c**) Showing the effect of RA on neuroectodermal differentiation of H9-hESCs in ACC system. (**b**) Flow cytometric analysis shows the effect of different concentration of RA at day 10. (**c**) Real-time PCR results show the temporal effect of 0.5 μM of RA. Error bars indicate the mean ± SEM ($n = 3$); *$P < 0.05$ versus "RA 10 days" sample. ACC, autogenic coculture system; FAD, F12 and Dulbecco medium; H9-hESC, H9-human embryonic stem cell; ES medium, embryonic stem cell medium; KSFM, keratinocyte serum-free medium; OD, optical density; RA, retinoic acid.

synergizes neuroectodermal differentiation, predominantly neural differentiation. However, the introduction of Activin at day 9, day 10, and day 11 augments ectodermal differentiation while inhibiting the neural differentiation effect of RA. The application of Activin at day 9 for a period of 3 days was found to be the most effective (Figure 3b). We found no substantial difference within different concentrations (10, 25, and 50 ng ml^{-1}) of Activin when applied at the optimized time point (Figure 3c). Thus, we were able to get around 85% ± 4.5 (SE) of ΔNp63 (+) cells from H9-hESCs at day 20 when treated with 0.5 μM of RAs for 20 days and 25 ng ml^{-1} of Activin for 3 days (from day 9 to day 11) in the ACC system (Figure 3d). However, the molecular mechanism of the downstream signaling pathway for the above mentioned effect requires further investigation.

Keratinocyte differentiation in the ACC system and characterization

After optimization of *in vitro* conditions, H9-hESCs were differentiated into keratinocytes in the ACC system (H9-Kert^{ACC}). H9-Kert^{ACC} were characterized at different time points (Figure 4a and b). We observed the higher expression of primitive epithelial marker K18 until day 20 from the start of

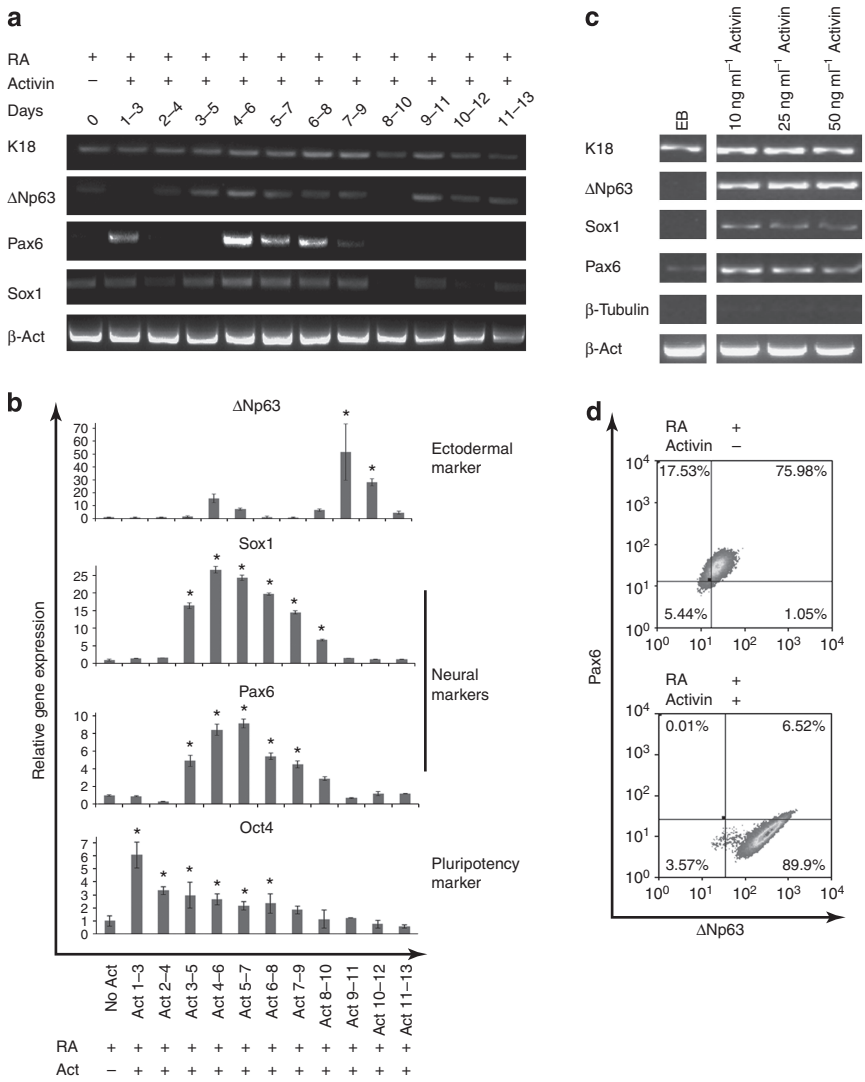

Figure 3. Temporal and concentration effect of Activin on H9-hESCs differentiation toward the neuroectodermal lineage in ACC system. (**a**, **b**) Qualitative and quantitative analyses show the temporal effect of Activin on H9-hESCs differentiation in the presence of RA at day 15. (**b**) Error bars indicate the mean ± SEM ($n = 3$); *$P < 0.05$ versus ''no Activin'' sample. (**c**) RT–PCR shows the effect of different concentrations of Activin at day 12. (**a**, **c**) β-Actin was taken as a loading control and run on a separate gel. (**d**) Flow cytometric analysis shows the effect of Activin in the presence of RA on the keratinocyte differentiation of H9-hESCs at day 20 in the ACC system. ACC, autogenic coculture system; Act, Activin; β-Act, β-actin; EB, embryoid body; H9-hESC, H9-human embryonic stem cell; RA, retinoic acid; RT–PCR, reverse transcriptase–PCR.

differentiation and then found it to have decreased at later stages (Figure 4b). Concurrently, epidermal precursor marker ΔNp63 and the basal keratinocyte marker of stratified epidermis, K14, were found to be increased steadily with time (Figure 4a and b). These results indicate that H9-hESCs *in vitro* follow the same hierarchy of embryonic keratinocyte differentiation. During embryogenesis, the K18 (+) cells in primitive epithelium are replaced by ΔNp63 (+) cells. Later, these progenitor cells give rise to basal keratinocytes (K14 (+) and integrin alpha 6 (+) cells) cells of stratified epidermis (Shalom-Feuerstein *et al.*, 2011). Alternatively, we found the progressive reduction of neural markers (Sox1, Pax6, and Nestin) and pluripotency markers (Oct4 and Nanog) during differentiation (Figure 4b). Characterization of H9-Kert^{ACC} at the protein level was done to confirm their keratinocyte lineage (Figure 4c–e). H9-Kert^{ACC} were able to undergo terminal differentiation and expressed suprabasal keratinocyte markers, such as involucrin and filaggrin in DSFM, supplemented with 1.5 mmol l^{-1} of calcium chloride. This suggests that H9-Kert^{ACC} possess characteristics of epidermal differentiating keratinocytes (Figure 4f).

In addition, we found that H9-Kert^{ACC} were able to maintain the high percentage of K14 (+) cells in serum-free medium (Figure 5a). H9-Kert^{ACC} were able to propagate for 10 passages without reaching the Hayflick limit (Figure 5b). However, we found the progressive shortening of telomere length and telomerase reverse transcriptase expression with increasing passage numbers (Figure 5c and d). Alternatively, we found no mouse embryonic fibroblast contamination in H9-Kert ^{ACC} (Figure 5e).

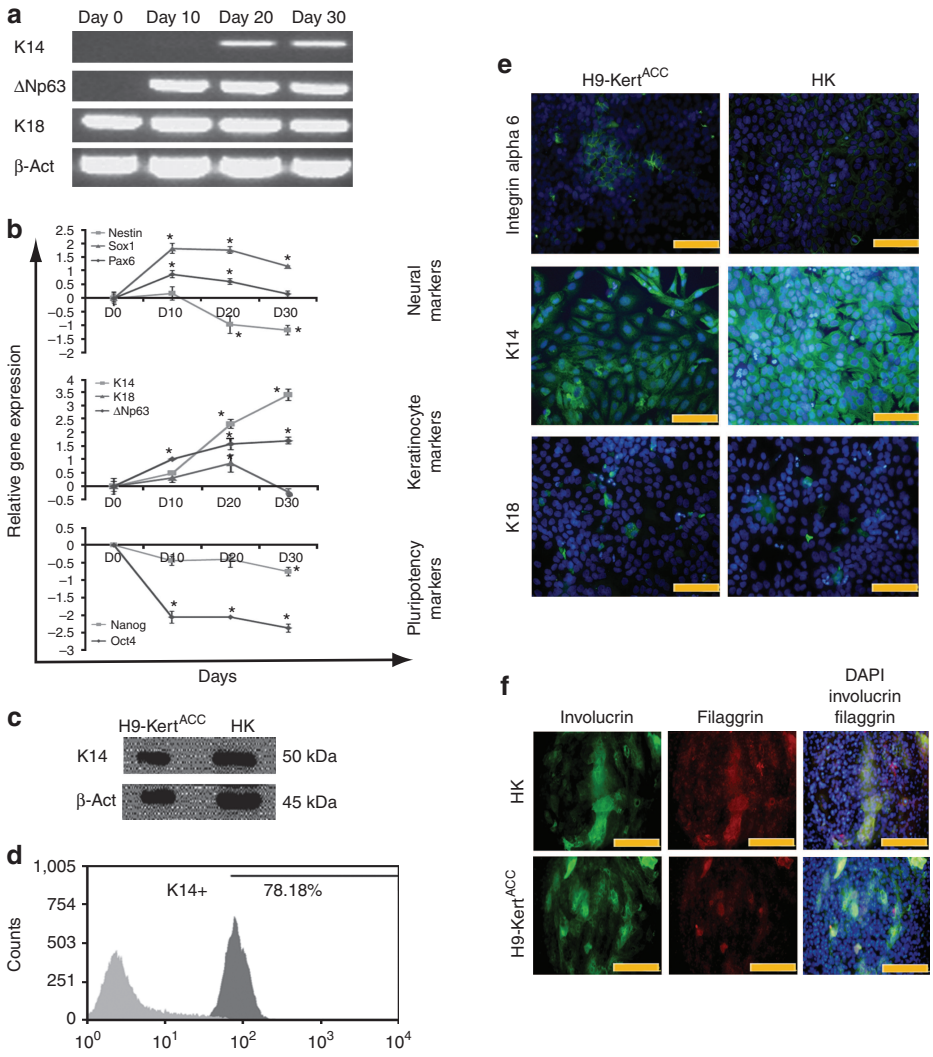

Figure 4. Characterization of H9-Kert^{ACC}. (**a**) Qualitative analysis shows keratinocyte markers kinetics. (**b**) Quantitative analysis shows the keratinocyte, neural, and pluripotency markers kinetics at different time points. Error bars = mean ± SEM ($n = 3$); *$P < 0.05$ versus 'D0' sample. (**c**) SDS-PAGE shows the expression of K14 protein by H9-Kert^{ACC} at day 30. (**d**) Flow cytometric analysis of H9-Kert^{ACC} shows the percentage of K14 (+) cells at day 30. (**e**, **f**) Immunocytochemistry of H9-Kert^{ACC} (passage 5) and HaCaT cell (positive control) for (**e**) integrin alpha 6, K14 protein (scale bar = 50 μm), and K18 (scale bar = 200 μm); (**f**) epidermal terminal differentiation markers (involucrin (green) and filaggrin (red)) (scale bar = 100 μm) in stratified area in high Ca^{2+} medium. β-Act, β-actin; DAPI, 4,6-diamidino-2-phenylindole; H9-Kert^{ACC}, H9-hESC-derived keratinocyte in an autogenic coculture system; HK, HaCaT cells.

Recently, Guenou *et al.* (2009) cocultured hESCs with mouse NIH 3T3 fibroblast cells for the induction of keratinocyte differentiation and were able to get 50–55% of K14 (+) cells in 40 days (which subsequently required sorting to achieve approximately 96% of K14 (+) cells). On the other hand, through our differentiation protocol in the ACC system, we were able to get 78% ± 7.5 (SE) of K14 (+) cells by day 30 and >95% of K14 (+) cells on subsequent culture. Our protocol does not require cell sorting to get the same percentage of K14 (+) cells as previously reported.

Establishment of the serum-free, feeder-free AFF system for keratinocyte differentiation

For more clinical relevance, we established an improved, defined, serum-free and autogenic feeder-free (AFF) system by extracting basement membrane-like ECM from H9-ebF. ECM from the basement membrane, which is also referred to as type IV matrix, mainly consists of collagen type IV, laminin, and proteoglycan. Basement membrane has an important role in keratinocyte differentiation (Miner *et al.*, 1997; Fleischmajer *et al.*, 2000), proliferation, and migration (Pouliot *et al.*, 2002). Collagen type IV is secreted predominantly by keratinocytes; however, it has been shown previously that transforming growth factor-β induces fibroblasts to secrete collagen IV (Lam *et al.*, 2004). Transforming growth factor-β and Activin share the same SMAD pathway. Alternatively, Activin was found to upregulate procollagen expression (cytoplasmic collagen) of fibroblasts. Subsequently, procollagen undergoes hydroxylation and glycosylation (post-translational modification) to form a triple helix and is extruded from the cells

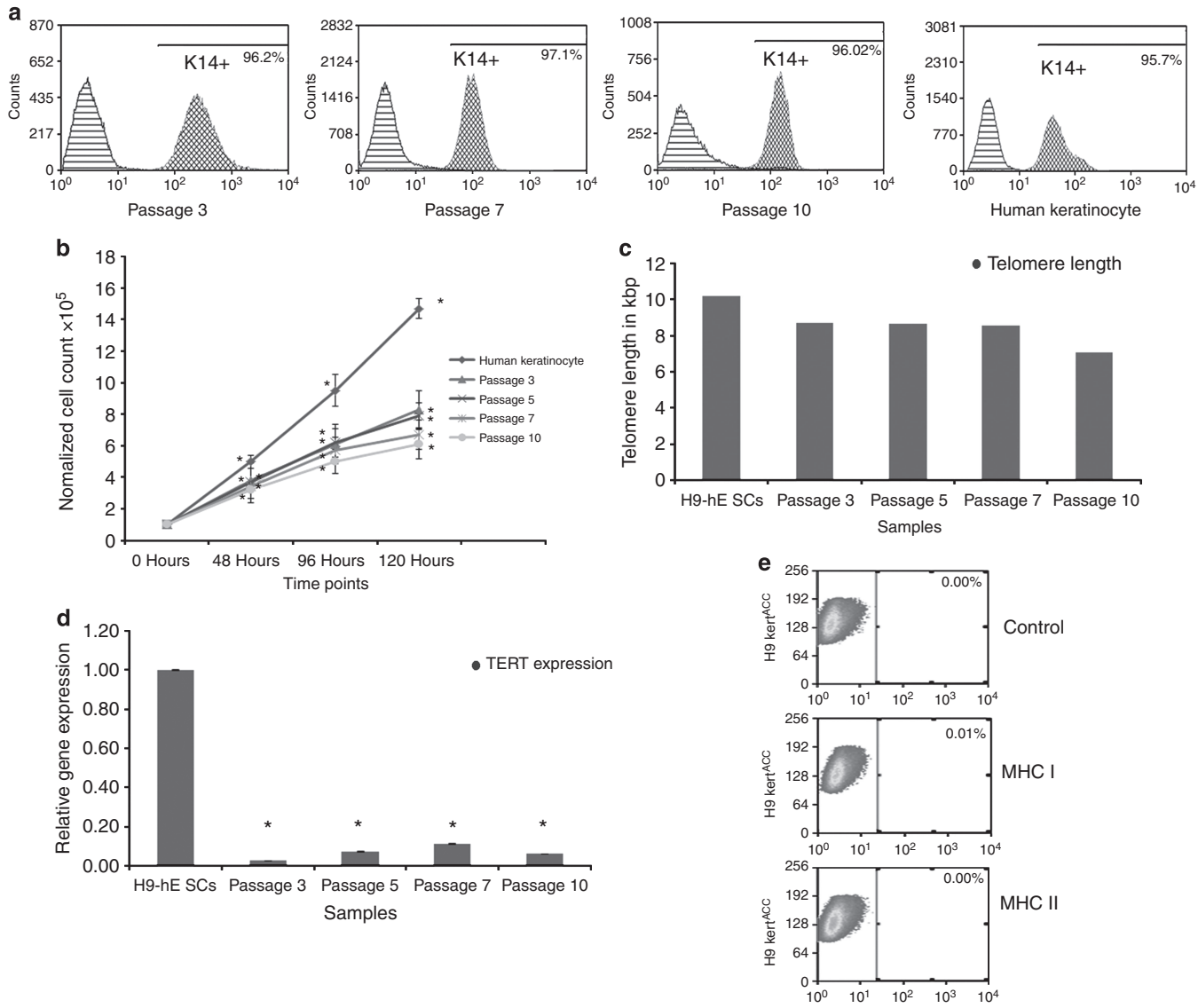

Figure 5. Characterization of different passages of H9-Kert^{ACC}. (**a**) Histogram data show the percentage of K14 (+) cells in different passages of H9-Kert^{ACC} using the flow cytometric analyses. Sample with isotype-specific or no primary antibody was taken as a control. (**b**) The cell count numbers of different passages of H9-Kert^{ACC}. (**c**, **d**) Quantitative analysis of (**c**) telomere length; (**d**) TERT expression. (**b**, **d**) Error bars = mean ± SEM ($n = 3$); (**b**) *$P < 0.05$ versus ''0 hours'' sample; (**d**) *$P < 0.05$ versus ''H9-hESCs'' sample. (**e**) Representative flow cytometric analysis of mouse MHC I and MHC II by H9-Kert^{ACC} passage 5. H9-hESC, H9-human embryonic stem cell; Human keratinocyte, HaCaT cells; H9-Kert^{ACC}, H9-hESC-derived keratinocyte in an autogenic coculture system; kbp, kilo base pair; MHC, major histocompatibility complex; TERT, telomerase reverse transcriptase.

(Tuan and Nichter, 1998; Niessen *et al.*, 1999; Derynck and Zhang, 2003; Mukhopadhyay *et al.*, 2007). We found similar results with H9-ebFs. H9-ebFs were able to secrete collagen Type IV in ECM substrate when exposed to crowding molecules like ascorbic acid and dextran sulfate supplemented with Activin for 3–5 days (Figure 6a). Alternatively, the exposure of H9-ebF to crowding molecules alone resulted in no collagen Type IV expression in ECM substrate. In addition, the application of Activin was also found to have enhanced ECM production from H9-ebF (Figure 6a and b).

Keratinocyte differentiation in the AFF system (H9-Kert^{AFF}) followed the same hierarchy of keratinocyte marker expression as H9-Kert^{ACC} (Figure 6c and d). We were able to get 89% ± 5.1 (SE) of K14 (+) by day 30 from the start of differentiation (Figure 6e). We found the AFF system as efficient as the xenogeneic Matrigel system for keratinocyte differentiation (Figure 6f). In addition, the immunofluorescence staining of H9-Kert^{AFF} showed higher expression of K14 and alpha integrin-6. Concurrently, we found substantially less expression of K18 similar as H9-Kert^{ACC} (Figure 6g). H9-Kert^{AFF} were able to grow until passage 7.

Recently, Metallo *et al.* used the xenogenic feeder-free system (Matrigel) for 6–10 days in a differentiation medium and then subcultured on gelatin-coated plates to get around 87% K14 (+) cells from hESCs by day 30. However, we

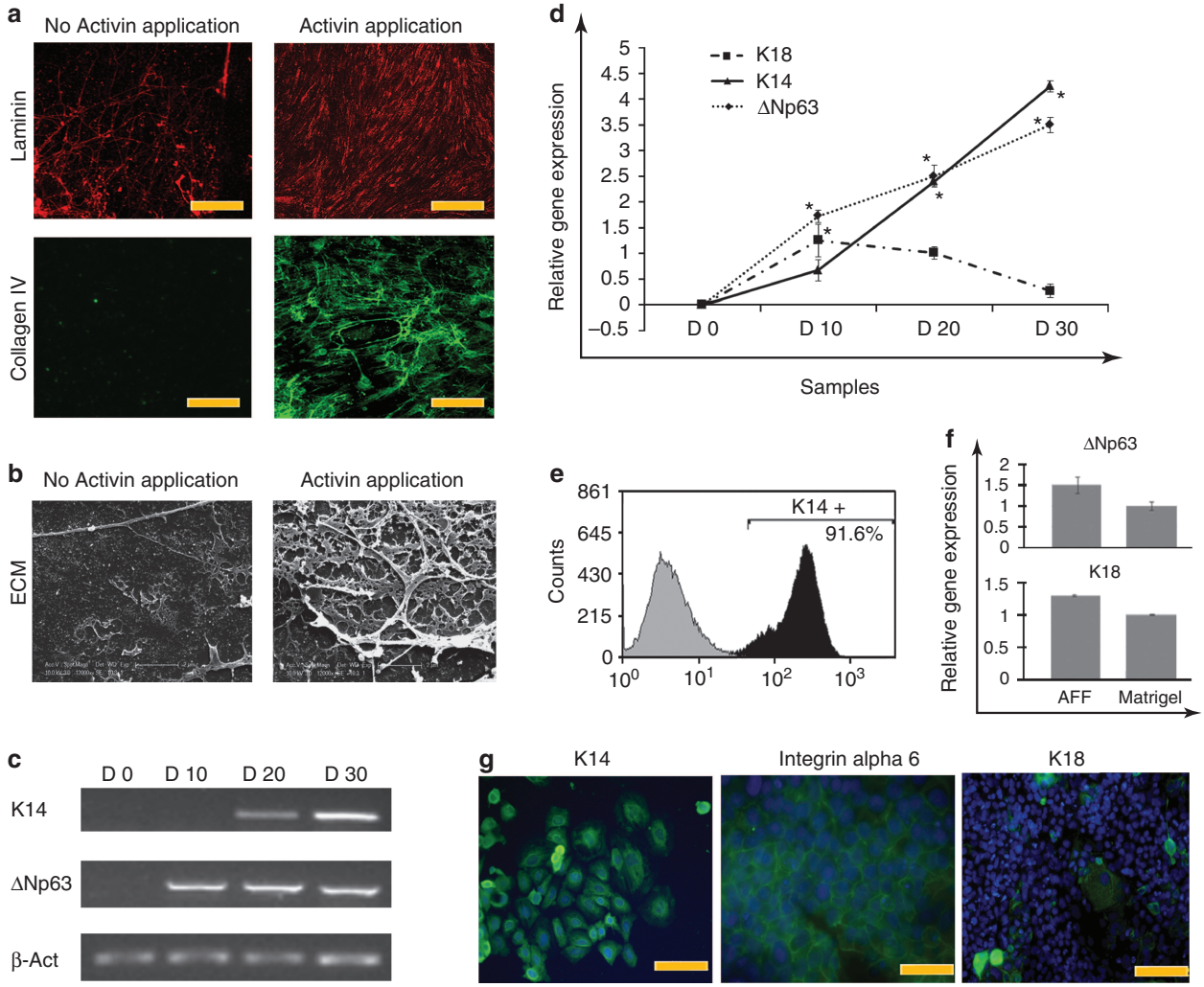

Figure 6. Characterization of AFF system and H9-KertAFF. (**a**, **b**) Characterization of the AFF system in the presence and/or absence of Activin. (**a**) Confocal microscopy images show the expression of laminin and collagen type IV. − = 100 ×. (**b**) SEM images of extracted ECM. (**c–f**) Characterization of H9-Kert^{AFF} (**c**, **d**) Qualitative and quantitative analyses of keratinocyte markers kinetics. (**c**) β-Act was run on a separate gel. (**d**) Error bars = mean ± SEM ($n = 3$); *$P < 0.05$ versus ''D0'' sample. (**e**) Flow cytometric analysis shows the K14 (+) cells at day 30. (**f**) Relative gene expression of keratinocyte progenitors in AFF and Matrigel systems at day 10. (**g**) Immunofluorescence staining of H9-Kert^{AFF} passage 5 for K14, integrin alpha 6, and K18 (scale bar = 100 μm). AFF, autogenic feeder-free system; β-Act, β-actin; D, day; ECM, extracellular matrix; H9-hESC, H9-human embryonic stem cell; H9-Kert ^{AFF}, H9-hESC-derived keratinocyte in an autogenic feeder-free system; SEM, scanning electron microscope.

induced differentiation of H9-hESCs on the AFF system and subcultured on human collagen type IV to obtain similar results.

Although we were able to differentiate hESCs into keratinocytes with the minimum use of materials from animal sources, DSFM and the medium used to derive H9-ebF and ECM were not totally free from animal components. Nevertheless, in our study, we have demonstrated that successful differentiation of expandable and highly enriched populations of keratinocytes is possible without using xenogeneic feeders or ECM. We also described the pivotal role of Activin in keratinocyte differentiation and in the production of specific types of ECM from fibroblasts. Therefore, the protocols described in this study are yet another step in bringing stem cell–based therapies closer to the clinic.

MATERIALS AND METHODS

Cell culture

H9-hESC lines were cultured and passaged as previously described (Itskovitz-Eldor *et al.*, 2000). Briefly, H9-hESCs were seeded on to an inactivated mouse embryonic fibroblast feeder cells in ES medium (1:1 DMEM/F-12, supplemented by 20% knockout serum replacement, 1% nonessential amino acid, 1 mM L-glutamine, 4 ng ml^{-1} fibroblast growth factor-2, and 0.1 mM β-mercaptoethanol (Sigma, St Louis, MO)). The medium was changed every day. All the media and components were obtained from Invitrogen (Carlsbad, CA) unless otherwise noted.

Generation of H9-ebF as feeder cells for the ACC system

For the establishment of the ACC system, H9-ebFs were obtained from H9-hESCs as described previously (Fu *et al.*, 2010). Briefly,

H9-hESCs colonies were dissociated into clumps by treatment with 1 mg ml^{-1} collagenase type IV and then transferred to nonadherent six-well culture plates in embryoid body medium (80% DMEM/F12 and 20% knockout serum replacer, supplemented with 1% non-essential amino acids, 1 mM L-glutamine, and 0.1 mM β-mecaptoethanol) for 5 days. Differentiated embryoid bodies were seeded on 0.1% gelatin-coated plates in a DMEM–fetal bovine serum (FBS) medium (high-glucose DMEM (Sigma) supplemented with 10% FBS (Biowest, Caille, Nuaille)) for 3 weeks. H9-ebFs were inactivated and plated onto the gelatin-coated 6-well plate of approximately 1.4×10^5 cells per well followed by H9-hESCs seeding on the next day. The H9-hESCs–H9-ebF ACC system was kept in ES medium for 2–3 days before the start of differentiation (Supplementary Figure S1 online).

Keratinocyte differentiation in the ACC system

For keratinocyte differentiation in the ACC system, ES medium was replaced by F12 and Dulbecco medium (mixture of 3:1 of DMEM and Ham's F12 media supplemented with 50 μg ml^{-1} ascorbic acid (Sigma), 2% FBS, 5 μg ml^{-1} insulin, 10 ng ml^{-1} recombinant human epidermal growth factor, and 0.5 μM of RA for 22–25 days. In addition, 25 ng ml^{-1} of Activin (catalog # 338-AC, R&D Systems, Minneapolis, MN) was introduced at day 9 until day 11 (3 days) from the day of differentiation. After 22–25 days, the differentiated cells were detached and seeded on human collagen type IV–coated flasks and kept in DSFM without Activin and RA (Supplementary Figure S2 online).

Extraction of ECM from H9-ebF for the AFF system

The AFF system was established with the modification of our previously published protocol (Fu *et al.*, 2011). Briefly, H9-ebF cells were seeded on six-well plates at a density of 2.5×10^5 per well and kept overnight in DMEM–FBS medium. Next day, the DMEM–FBS medium was replaced with Crowding medium (DMEM) supplemented with 0.5% FBS, 50 mg ml^{-1} ascorbic acid (Sigma), 100 mg ml^{-1} of dextran sulfate (Sigma), and 25 ng ml^{-1} recombinant human Activin for 3–5 days followed by overnight incubation at −80 °C. Next day, the plate was thawed at room temperature (RT) and each well was rinsed with phosphate-buffered saline three times to get rid of cell debris to establish the AFF system. H9-hESCs were seeded onto the AFF system in an animal component–free TeSR 2 (StemCell Technology, Vancouver, BC, Canada) medium for 2–3 days before the start of differentiation (Supplementary Figure S3 online).

Keratinocyte differentiation in the AFF system

For keratinocyte differentiation of H9-hESCs in the AFF system, animal-free TeSR 2 medium was replaced by differentiation medium (DSFM supplemented with 1 μM of RA) and applied for 10–12 days. The differentiated cells were subcultured on human collagen type IV–coated plates and kept in DSFM without RA.

Keratinocyte cell lines HaCaT were taken as control. Control cell line was grown in DMEM–FBS medium.

Reverse transcriptase–PCR and real-time PCR analysis

For reverse transcriptase–PCR, cells were subjected to RNA extraction using the RNeasy Mini Kit (Qiagen, Valencia, CA), following the manufacturer's instructions. β-Actin served as a loading control.

For real-time PCR, the reactions were performed using the SYBR Green PCR Master Mix System (Applied Biosystems, Foster City, CA) and processed on the StepOnePlus real-time PCR system (Applied Biosystems). Expression of β-actin was used as the reference gene. The expression level of each target gene was then calculated as $2^{\Delta\Delta Ct}$ as described previously (Livak and Schmittgen, 2001). A melting curve was used to verify the PCR products (Supplementary Table S4 online).

ELISA analysis

ELISA was performed using the DuoSet ELISA Development System (catalog # DY251, R&D systems) as per the manufacturer's instructions. Absorbance of each well was measured at 450 nm.

Western blotting

Cell extracts were prepared in RIPA lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 1X EDTA-free complete protease inhibitor cocktail. The sample was centrifuged and the supernatant was collected. Samples were then boiled for 5 minutes at 90 °C, electrophoresed on 12% SDS-polyacrylamide gel, and transferred onto polyvinylidene difluoride membrane. The membrane was blocked for 1 hour with 5% nonfat dry milk in phosphate-buffered saline with Tween (0.1% Tween 20) and probed with the primary antibody (1:1000) (Supplementary Table S5 online) overnight at 4 °C. Blots were then incubated with peroxidase-conjugated secondary antibody (1:5000) (Supplementary Table S5 online) for 1 hour at RT, and were developed by enhanced chemiluminescence (Pierce, Rockford, IL).

Immunofluorescence, confocal microscopy, and flow cytometry

Staining was done as described previously (Yang *et al.*, 2009). Briefly, samples were fixed for 15 minutes in 4% paraformaldehyde at RT, permeabilized with 0.4% Triton X-100/phosphate-buffered saline (if required) for 10 minutes, and blocked with phosphate-buffered saline containing 4% BSA (Sigma) for 1 hour at RT. The cells were incubated overnight at 4 °C with the primary antibody (1:100) (Supplementary Table S5 online). Samples were washed and followed by the incubation in secondary antibody (1:100) (Supplementary Table S5 online) for 0.5 hours at RT in the dark.

Scanning electron microscope

The samples were fixed in 10% formalin and then dried in ascending alcohol concentrations. Samples were dehydrated in a critical point dryer and sputter coated with gold under vacuum and examined in scanning electron microscope (XL30 FEG SEM, FEI, Philips, Eindhoven, The Netherlands).

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay

For MTS assay, the CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega, Southampton, UK) was used as per the manufacturer's instructions. Absorbance at 490 nm was recorded.

Telomere length assay

Telomere length assay was performed using the Telo TAGGG telomere Length Assay Kit (Cat # 12 209 136 001, Roche Diagnostic, Castle Hill, NSW, Australia) according to the manufacturer's instructions.

Statistical analysis

For statistical analysis, one-way analysis of variance *post hoc* tests (Tukey and Bonferroni) and Student's *T*-test were used. A value of $P<0.05$ was considered as significantly different.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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